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Original Article

Human placental extract improves liver cirrhosis in mice with regulation of macrophages and senescent cells

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ABSTRACT

Introduction: Cirrhosis is a disease with poor prognosis that requires the development of a novel therapeutic approach alternative to liver transplantation. In this study, we focused on the placenta and aimed to clarify the effects of human placental extract (HPE) on cirrhosis.
Methods: A mouse model of carbon tetrachloride-induced cirrhosis was used to evaluate the effect of HPE administration subcutaneously and compared with the control group (n = 8 for each group). In vitro and *in vivo*, real time-PCR and immunostaining were performed for HPE mechanistic analysis. Spatial transcriptomics was also performed for detailed analysis of the effect of HPE on cirrhosis.
Results: HPE administration improved serum ALT levels compared to control mice. Furthermore, there was a decrease in the number of senescent cells in the liver and the mRNA levels of secrete senescence-associated secretory phenotype factors *and Cdkn2a (p16). In vitro,* HPE induced macrophage polarization to the anti-inflammatory M2 phenotype. Spatial transcriptomics was also performed to analyze the underlying anti-inflammatory mechanism. The results showed that HPE strongly polarized macrophages to the M2 phenotype, especially in macrophage-rich regions in the liver. Gene expression pathway analysis using spatial transcriptomics also revealed the possibility of improving senescent cell-derived inflammation via mitochondrial function.
Conclusions: HPE improves serum ALT levels via anti-inflammatory mechanisms in macrophages and senescent cells. HPE serves as a novel agent for cirrhosis treatment.

Abbreviations: ALT, alanine aminotransferase; CCl_4, carbon tetrachloride; γ-H2AX, gamma-H2AX; HPE, human placental extract; IL, interleukin; LC, liver cirrhosis; ROI, regions of interest; RT-PCR, real-time PCR; PAI-1, plasminogen activator inhibitor type 1; TNF, tumor necrosis factor; SASP, secrete senescence-associated secretory phenotype; tSNE, t-distributed stochastic neighbor embedding.

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1. Introduction

Chronic liver injury caused by hepatitis virus infection, alcohol abuse, lifestyle disorders, and autoimmunity progressively leads to liver fibrosis, resulting in cirrhosis. Cirrhosis is a lethal condition that can lead to portal hypertension, liver failure, and hepatocellular carcinoma [1,2]. There are approximately 112 million patients with cirrhosis and 10 million with decompensated cirrhosis worldwide, resulting in 1.32 million deaths annually [3]. The main treatment for chronic liver damage is to remove the cause of the liver disease such as anti-viral agents. The only radical treatment for cirrhosis is liver transplantation; however, due to its invasiveness, shortage of donors, lifelong use of immunosuppressive agents, and economic burden, the number of patients with cirrhosis who can receive liver transplantation is extremely limited [4,5]. These issues show that there is a high unmet medical need for cirrhosis, and a novel therapeutic approach for cirrhosis is required.

Human placental extract (HPE) (Laennec®) is obtained from the chemical modification of the human placenta. HPE is purely a collection of extracts from the placenta; therefore, HPE contains several factors derived from placenta such as cytokines, chemokines, growth factors, and extracellular vesicles. HPE can be administered subcutaneously, and only a few adverse events have been reported. The effect of HPE has been reported to improve liver damage in chronic hepatitis [6]. HPE has also been reported to show anti-fibrosis, anti-lipogenesis, and liver regeneration properties [7–10] and is expected to be applied in the treatment of cirrhosis.

Cyclo-trans-4-L-hydroxyprolyl-L-serine is a component of HPE that may contribute to improving liver dysfunction by downregulating the expression of intercellular adhesion molecule-1 and tumor necrosis factor (TNF)-ά [8]. Cyclo-trans-4-L-hydroxyprolyl-L-serine is also reported to contribute to apoptosis inhibition by downregulating the expression of caspase 3 [9]. Furthermore, pyroglutamic acid in HPE promotes DNA synthesis via the MAPK pathway [10]. Thus, although HPE is expected to be indicated for the treatment of cirrhosis, its therapeutic mechanism remains unclear. Therefore, it is important to elucidate its mechanism and apply HPE to the treatment for cirrhosis.

In this study, we investigated the effects of HPE in cirrhotic. Specifically, we performed spatial transcriptomics to examine the mechanisms underlying the role of HPE in cirrhosis.

2. Materials and methods

2.1. Mice

All animal experiments were conducted in compliance with the pertinent regulations. The experimental protocol was approved by the Institutional Animal Care and Committee at Niigata University (SA01211). C57BL/6 male mice were purchased from Charles River Laboratories (Yokohama, Japan). The animals were housed in a specific pathogen-free environment under standard conditions with a 12-h day/night cycle and access to food and water *ad libitum*.

2.2. Obtaining HPE

HPE (Laennec®, lot number: 224939) was provided by Japan Bio Products Co., Ltd. All HPE had the same lot number.

2.3. Generation of a mouse model of carbon tetrachloride-induced liver injury

Cirrhosis was induced in 8-week-old male mice via intraperitoneal injection of 1.0 mL/kg carbon tetrachloride (CCl_4; Wako, Osaka, Japan) twice a week for 8 weeks. CCl4 was dissolved in corn oil (Wako) before use.

2.4. HPE administration and analysis

Cirrhosis was induced in 8-week-old mice. After 6 weeks of CCl4 injection, 0.10 mL HPE was administered to cirrhotic mice subcutaneously twice a week for 3 weeks. The mice (17-week-old mice) were analyzed after 9 weeks.

2.5. Reverse transcription real time- PCR

Total RNA was reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen, Tokyo, Japan). Gene expression analysis was performed using pre-validated QuantiTech primers (Supplemental Table 1) with QuantiTech SYBR reagent (Qiagen). Real-Time PCR was performed using a Step-One-Plus Real-Time PCR System (Applied Biosystems, Foster City, USA). *GAPDH* was used as an internal control. The fold change in relative gene expression compared to the control was calculated using the $2^{-\Delta\Delta CT}$ method.

2.6. Immunostaining

For immunostaining, 10 % formalin-fixed tissues were cut into 3-μm-thick sections. Heat-mediated antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0). For DAB staining, sections were blocked with 3 % hydrogen peroxide (Wako), Avidin/Biotin Blocking Solution (Thermo Fisher Scientific, MA, USA), Blocking One Solution (Nacalai Tesque, Kyoto, Japan), and Mouse-on-Mouse IgG Blocking Solution (Thermo Fisher Scientific), followed by incubation with primary antibodies (Supplemental Table 2) overnight at 4 °C. Species-specific anti-IgG biotinylated antibodies were used for detection. The slides were stained using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA) and DAB substrate. Nuclei were counterstained with hematoxylin. Images were acquired using an HS All-in-One fluorescence microscope (BZ-X810) (Keyence, Osaka, Japan).

2.7. Spatial transcriptomics analysis

For analysis, 10 % formalin-fixed livers were cut into 3-μm-thick sections. Liver samples were subjected to spatial transcriptomics, performed by Eurofins Genetic Lab Co., Ltd. (Hokkaido, Japan). After immunostaining for cytokeratin8/18 (blue), F4/80 (red), and Syto 83 (green), the regions of interest (ROI) were set as references for each staining. Gene expression was comprehensively analyzed in each ROI.

2.8. Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences version 25 (International Business Machines Corporation, Tokyo, Japan) and Microsoft Excel (Microsoft, Washington, DC, USA). Data are presented as the mean ± SD, and the data analyzed were normally distributed. The results were assessed using Student's *t*-test. Differences between groups were analyzed using one-way or two-way anova. Differences were considered significant at $p < 0.05$.

Further descriptions of the experimental procedures are provided in the supplemental information.

3. Results

3.1. HPE administration improved serum ALT levels and reduced the number of senescent cells in the livers of cirrhosis model mice

To examine the therapeutic effects of HPE, HPE was subcutaneously administered to mice (n = 8 per group). A simplified timeline is shown in Fig. 1A. The dose and route of HPE administration were determined in reference to previous reports and drug information of HPE. As no problems were observed during the tolerance check, HPE was administered twice a week for 3 weeks at a dose of 0.10 mL. Evaluation of hepatobiliary enzyme after HPE administration showed that serum alanine aminotransferase (ALT) levels were lower in the HPE group compared to the control group (Fig. 1B). Evaluation of cellular senescence showed that the number of p16-positive cells, gamma-H2AX (γ-H2AX) - positive cells, and Interleukin-6 (IL-6) - positive cells in the liver decreased in the HPE group compared to the control group (there was no difference in the number of p21-positive cells) (Fig. 1C, Supplemental Fig. 1, Supplemental Fig. 2, Supplemental Fig. 3). Regarding the evaluation of liver fibrosis, the results of Sirius Red staining of the liver were 2.77 ± 0.69 % in the control group and 2.32 ± 0.28 % in the HPE group (Supplemental Fig. 4), which showed no significant improvement but tendency to decrease in the HPE group compared to the control group. These findings indicate that HPE suppresses inflammation and reduces cellular senescence in cirrhosis.

3.2. HPE administration downregulated the mRNA levels of secrete senescence-associated secretory phenotype factors and Cdkn2a (p16) in the livers of cirrhosis

In vivo examination revealed that HPE suppressed liver inflammation and reduced the number of senescent cells in the liver. To elucidate the underlying mechanisms, we evaluated mRNA expression changes in the livers of cirrhotic model mice. We performed a comprehensive real-time PCR of secrete senescence-associated secretory phenotype (SASP) factors including inflammation (Fig. 2A), cellular senescence (Fig. 2B), and liver fibrosis (Supplemental Fig. 5). The results showed that the mRNA levels of *IL-6, plasminogen activator inhibitor type 1 (PAI-1), CCl2, tumor necrosis factor (TNF) -α, and Cdkn2a (p16)* decreased in the HPE group. These findings suggested that HPE administration reduced inflammation and cellular senescence in the liver.

3.3. HPE can polarize macrophages to the anti-inflammatory phenotype

In vivo results showed significant improvement in serum ALT levels in the HPE administration group. To elucidate the anti-inflammatory and regenerative mechanism in the liver, in addition to a comprehensive real-time PCR analysis, we focused on macrophages and hepatocytes that are relatively abundant in the liver and contribute to the control inflammation and regeneration in the liver *in vitro*. Briefly, HPE was added to primary culture-derived macrophages and cell line-derived hepatocytes *in vitro*, and changes in mRNA levels 48 h after HPE addition were analyzed. Regarding macrophages, HPE downregulated the mRNA levels of the markers of inflammatory M1 phenotype (tumor necrosis factor [*TNF*]-α and induced nitric oxide synthase [*iNOS*]) and upregulated the mRNA levels of M2 phenotype markers (*Fizz-1, Ym-1, and CD206*) (Fig. 2C). Regarding hepatocytes, no significant changes in mRNA levels after HPE addition but tendency to upregulate the mRNA levels of hepatocytes growth factor and oncostatin M (Supplemental Fig. 6). These findings suggest that HPE may induce macrophage polarization toward the M2 phenotype, suppressing inflammation in the liver *in vivo*.

3.4. HPE administration strongly polarized macrophages into the M2 phenotype in the macrophage-rich regions of the liver

We revealed that macrophages contribute to improving serum ALT levels by HPE administration; therefore, we focused on macrophages in the liver and performed a comprehensive gene expression and spatial transcriptomics analysis. Immunostaining with cytokelatin8/18 and F4/80 was performed in the liver tissue of the control and HPE administration groups to recognize hepatocytes and macrophages, respectively, and ROI were set on the tissue (Fig. 3A). To examine the influence of macrophages for the therapeutic mechanism, ROI were set in regions where macrophages were abundant or not, and gene expression within the ROI was analyzed. A representative ROI with abundant macrophages in the control group is shown in Fig. 3B and that in the HPE administration group is shown in Fig. 3C. At least three ROI were analyzed for each region, and genes with significant changes were extracted ($p < 0.05$). A t-distributed stochastic neighbor embedding (tSNE) map was used to visualize gene expression levels in the control and HPE groups. Gene expression levels in the tSNE map are separated into the control (black dotted circle) and HPE groups (red dotted circle) (Fig. 3D). Based on the separation of the tSNE map, a volcano plot was constructed for the genes expressed in the ROI of the macrophage-rich regions in the control group compared to those in the HPE group (Fig. 4A). The raw volcano plot data are presented in Supplemental Table 3. Among the top 10 enriched genes, the CYP2 family [11–13], which polarizes macrophages into the anti-inflammatory M2 phenotype, ranked first, third, fourth, fifth, and sixth. Furthermore, ribonuclease-4 and angiogenin [14–16], which are related to cell proliferation, were ranked second and seventh (Fig. 4B). Similarly, a volcano plot was also constructed for genes expressed in the ROI of macrophage-poor regions (Fig. 4C). Raw volcano plot data are presented in Supplemental Table 4. In the top 10 enriched genes of the macrophage-poor ROI, the CYP2 family was only ranked second, and no other notable genes were observed (Fig. 4D). These results suggested that macrophages in the rich regions may have a stronger ability to change their polarity into the anti-inflammatory M2 phenotype than those in the poor regions.

3.5. Pathways related to mitochondrial function and inflammation were enriched in the HPE administration group

Spatial transcriptomics analysis revealed that genes expressed in the livers of the control and HPE administration groups were significantly different. Therefore, pathway analysis of the genes expressed in the macrophage-rich ROI was performed. The raw pathway data are presented in Supplemental Table 5. Pathway intensity was scored between 0 and 1 (enrichment score), and the top 40 factors, to the absolute values of the scores, were extracted (Fig. 4E). Eleven pathways related to mitochondrial function, such as “mitochondrial biogenesis,” had high pathway coverage. The pathways “SASP” and “signaling by interleukins” were also included, which are strongly related to anti-inflammation. Furthermore, only one pathway related to MAPK signaling was included at the 35th position of the analysis. These results support the anti-senescent and anti-inflammatory effects of HPE in the liver and suggest that HPE administration may improve serum ALT levels via these mechanisms.

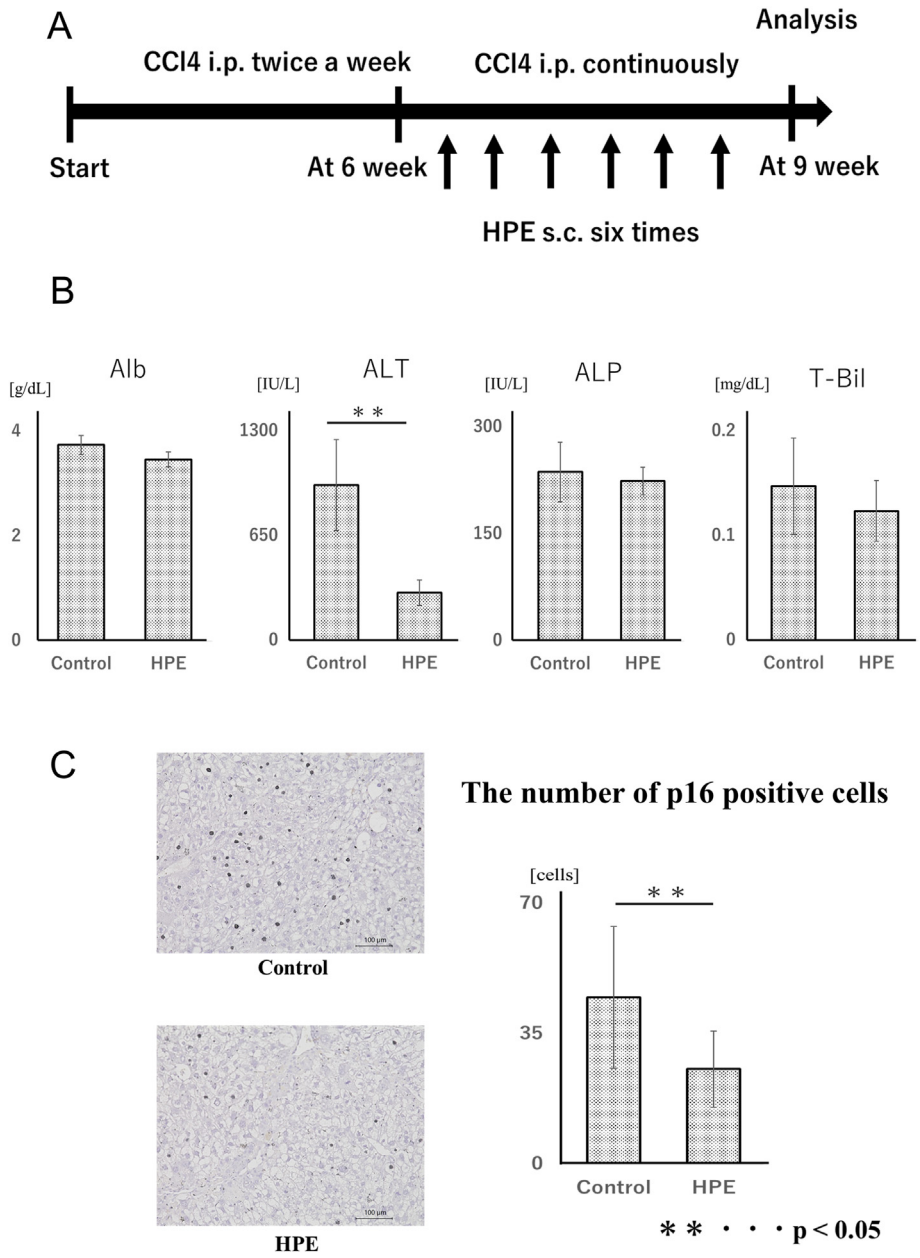

Fig. 1. Therapeutic effect of human placental extract (HPE) on cirrhosis in mice. (A) Schematic representation of our *in vivo* study. (B) Serum levels of albumin (Alb), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin (T-Bil) after HPE administration. Data are presented as the mean ± SD. n = 8 in each group. $p < 0.05$ (ALT, HPE administration group compared to the control group). (C) Immunostaining for p16 in the mouse liver. n = 8 for each group. Data are presented as the mean ± SD, $p < 0.05$ (HPE administration group compared to the control group).

4. Discussion

Despite developments in medicine, there are still many deaths due to cirrhosis worldwide, and novel treatments are needed to resolve this problem [1,3,17]. Therefore, we examined the effects of HPE on CCl4-induced cirrhosis in mouse models *in vitro* and *in vivo*. As HPE is extracted from the human placenta, it is assumed to contain many placenta-derived trophic factors [18,19]. Although the composition of HPE is not yet known, it may have anti-inflammatory and regenerative effects according to previous studies [6–10]. Therefore, HPE may represent a novel and promising avenue for the treatment of cirrhosis.

A cirrhosis mouse model with intraperitoneal CCl4 administration continuously was used in this study [20,21]. Although this model dose not show a fluctuation in albumin or bilirubin levels in the serum, it has been used in many studies as a model of mild to moderate cirrhosis and was presumed to be appropriate for the validation of the effect of HPE in this study. *In vivo*, HPE treatment improved serum ALT levels. Based on this result, we postulated two mechanisms: the anti-inflammatory effect mediated by macrophages in the liver and the suppression of inflammation derived from senescent cells. Regarding the macrophage-mediated response, macrophages have diverse phenotypes and plasticity and are characterized by changing their phenotype depending on the surroundings [22]. There are two representative and well-known macrophage phenotypes: the classically activated M1 phenotype, which is an inflammatory macrophage, with iNOS, TNF-α, and CCl2 as major markers, and the alternatively activated M2

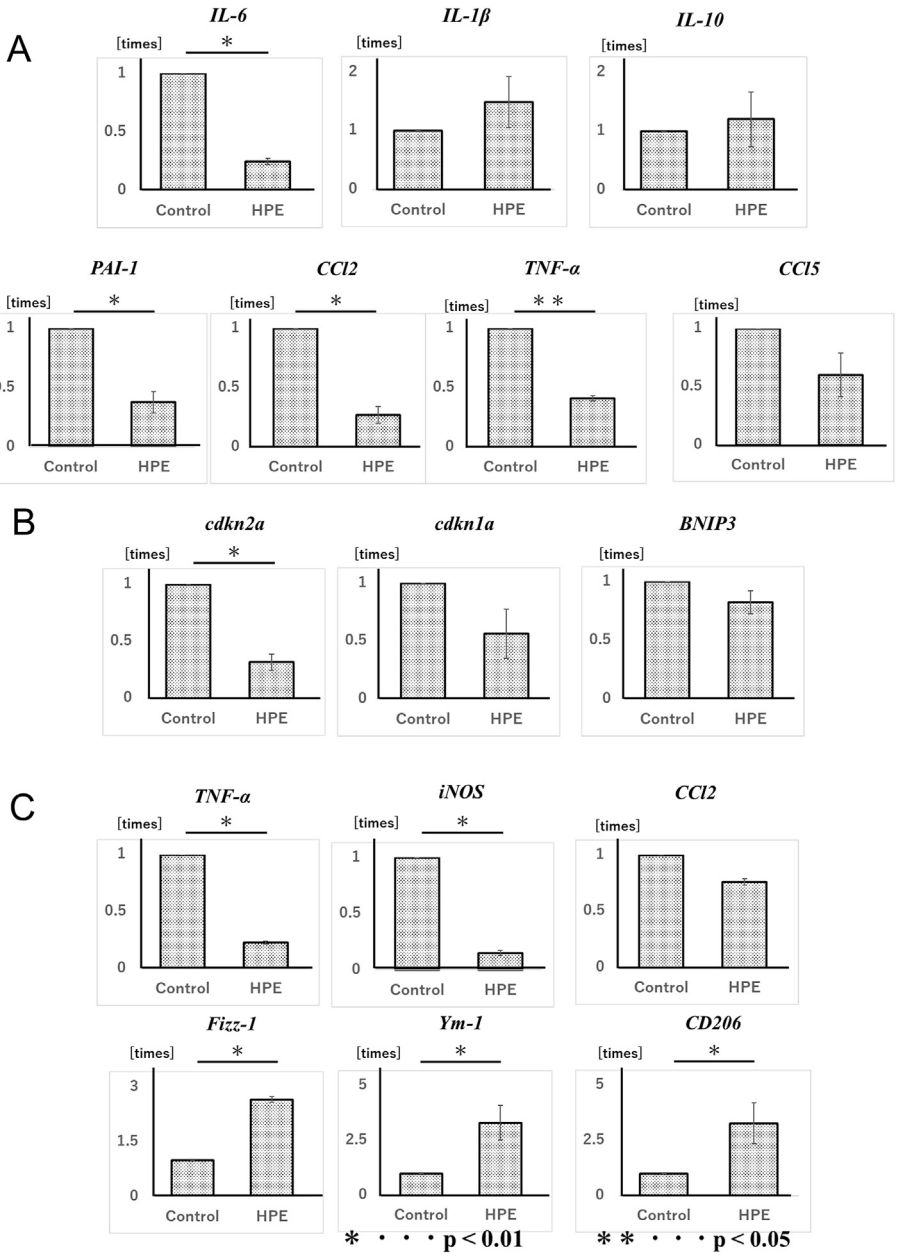

Fig. 2. Changes in the mRNA expression in the whole liver of mice after human placental extract (HPE) administration *in vivo* and in the macrophages after HPE addition *in vitro*. Data are presented ratio to the standards as the mean ± SD. n = 8 for each group. (A) Changes in the mRNA expression of genes related to inflammation and senescence-associated secretory phenotype (interleukin-6 [IL-6], interleukin-1β [IL-1β], interleukin-10 [IL-10], plasminogen activator inhibitor type 1 [PAI-1], CCl2, tumor necrosis factor-α [TNF-α], and CCl5) *in vivo*. $p < 0.01$ (IL-6, PAI-1, CCL2 and CCl5; HPE compared to the Control). (B) Changes in the mRNA expression of genes related to cellular senescence (Cdkn2a, Cdkn1a, and BNIP3). $p < 0.01$ (Cdkn2a; HPE compared to Control). (C) *In vitro* changes in the mRNA expression of genes related to macrophage markers of the M1 phenotype (tumor necrosis factor-α [TNF-α], induced nitric oxide synthase [iNOS], and CCl2) and related to macrophage markers of the M2 phenotype (Fizz-1, Ym-1, and CD206). $p < 0.01$ (TNF-α, iNOS, Fizz-1, Ym-1, and CD206; HPE compared to Control).

phenotype, which is an anti-inflammatory macrophage, with Fizz-1, Ym-1, and CD206 as its major markers [22,23]. During tissue injury activation, macrophages polarize to the M1 phenotype to activate inflammation. In contrast, macrophages polarize to the M2 phenotype during tissue injury stabilization and contribute to tissue repair [23]. *In vitro*, HPE downregulated the mRNA levels of M1 macrophage markers and upregulated M2 macrophage markers, changing macrophage polarity to the M2 phenotype. Thus, HPE may improve serum ALT levels through its anti-inflammatory effects on macrophages. p16-positive cells are representative senescent cells that become incapable of cell division or undergo apoptosis due to mitochondrial dysfunction [24–26]. Regarding the senescent cells-mediated response, senescent cells secreted SASP including IL-6, PAI-1, CCl2, CCl5, and TNF-α which have negative effects on the surrounding tissue or cause damage to several organs [27–30]. Our *in vivo* results showed that although there was no differences in the number of p21-positive cells, the number of senescent cells (p16-positive cells, γ-H2AX-positive cells, and IL-6 positive cells) decreased in the HPE administration group, and our *in vitro* results showed that mRNA levels of *IL-6, PAI-1, CCl2, TNF-α, and Cdkn2a (p16)* were downregulated in the whole liver of the HPE administration group. Thus, the SASP derived from

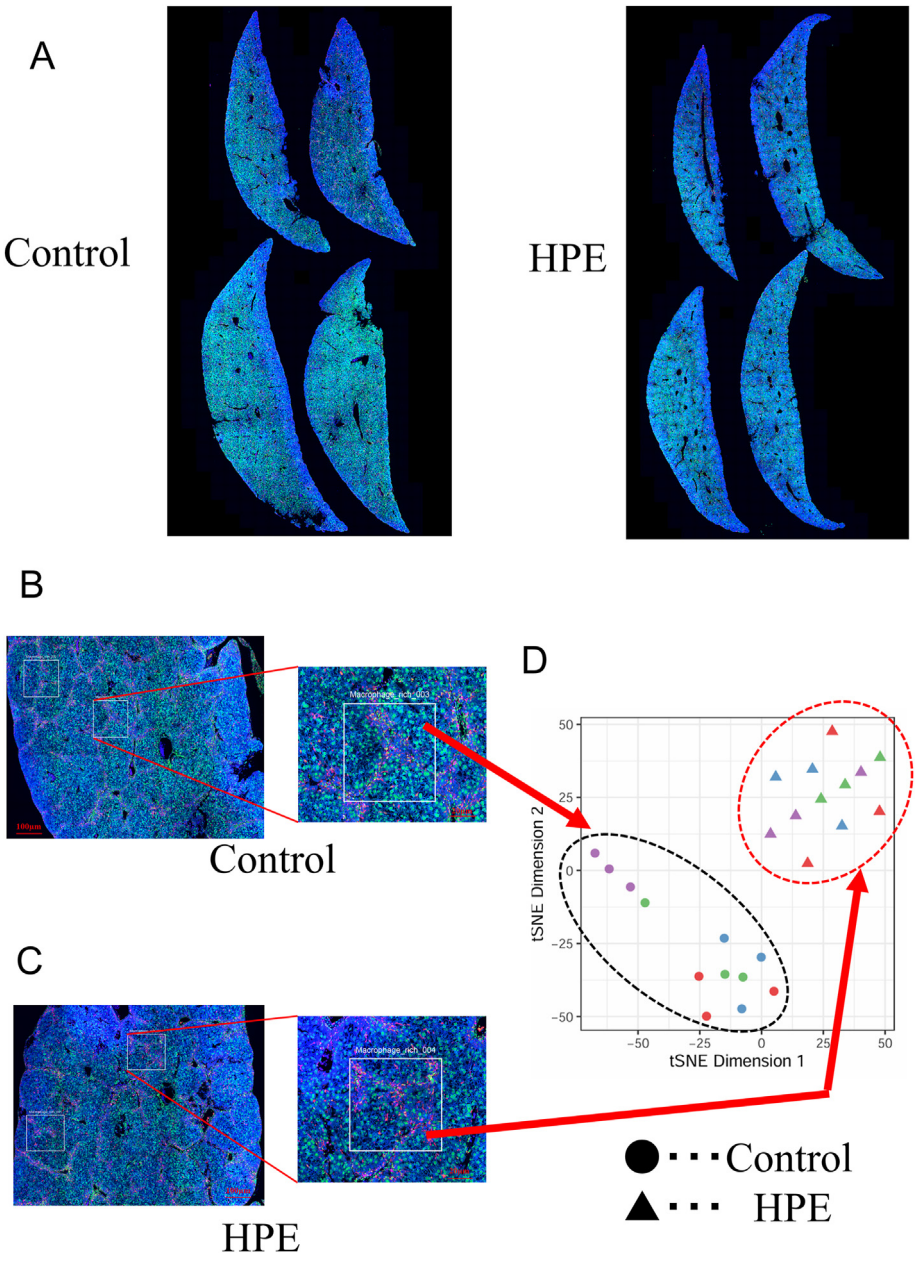

Fig. 3. Immunostaining of the livers for spatial transcriptomics analysis. (A) Immunostaining for cytokelatin8/18 and F4/80 in the livers of mice in the control and human placental extract (HPE) administration groups. Green: hepatocytes; purple: macrophages; blue: nuclei. Regions of interest were set in the tissues of macrophage-rich or macrophage-poor regions. (B and C) A representative macropphage-rich regions of interest (ROI) with fluorescent immunostaining for cytokelatin8/18 and F4/80 in the livers of mice in the control and HPE administration groups (Green: hepatocytes; purple: macrophages; blue: nuclei) (Scale bar in the left images of each are 100 μm and that in the right images of each are 20 μm). (D) A t-distributed stochastic neighbor embedding map of the genes expressed in the liver of the control (black dotted circle) and HPE administration groups (red dotted circle). Gene expression in the control group is represented as circle plots, while that of the HPE administration group is represented as triangle plots.

senescent cells was reduced by HPE administration, leading to improved serum ALT levels. Therefore, HPE may have therapeutic effects via macrophages and senescent cells.

Spatial transcriptomics was performed considering the anti-inflammatory mechanism of macrophages and senescent cells. Spatial transcriptomics is a method used to comprehensively analyze gene expression in tissues. It is also useful for extracting genes expressed at a specific region in the tissue and allows for the comparison of genes expressed in different regions [31–33]. Our spatial transcriptomics analysis in this study revealed the following three points: (i) the genes expressed in the control and HPE administration groups were clearly different according to the tSNE map, (ii) the expression of the CYP2 family was extremely high in the macrophage-rich regions, and (iii) pathway analysis showed that many pathways related to anti-inflammation and mitochondrial function were observed. Regarding point (i), the differences observed in the tSNE map strongly supported the therapeutic effects of HPE. Regarding point (ii), CYP2 is responsible for polarizing macrophages to the M2 phenotype [11–13]. Since CYP2 was highly expressed in macrophage-rich regions and not in macrophage-poor regions, macrophages might play a major role in the anti-inflammatory process. Regarding point (iii), some pathways related to interleukins were enriched, which might reflect the anti-inflammatory effect observed in this study. Furthermore, many

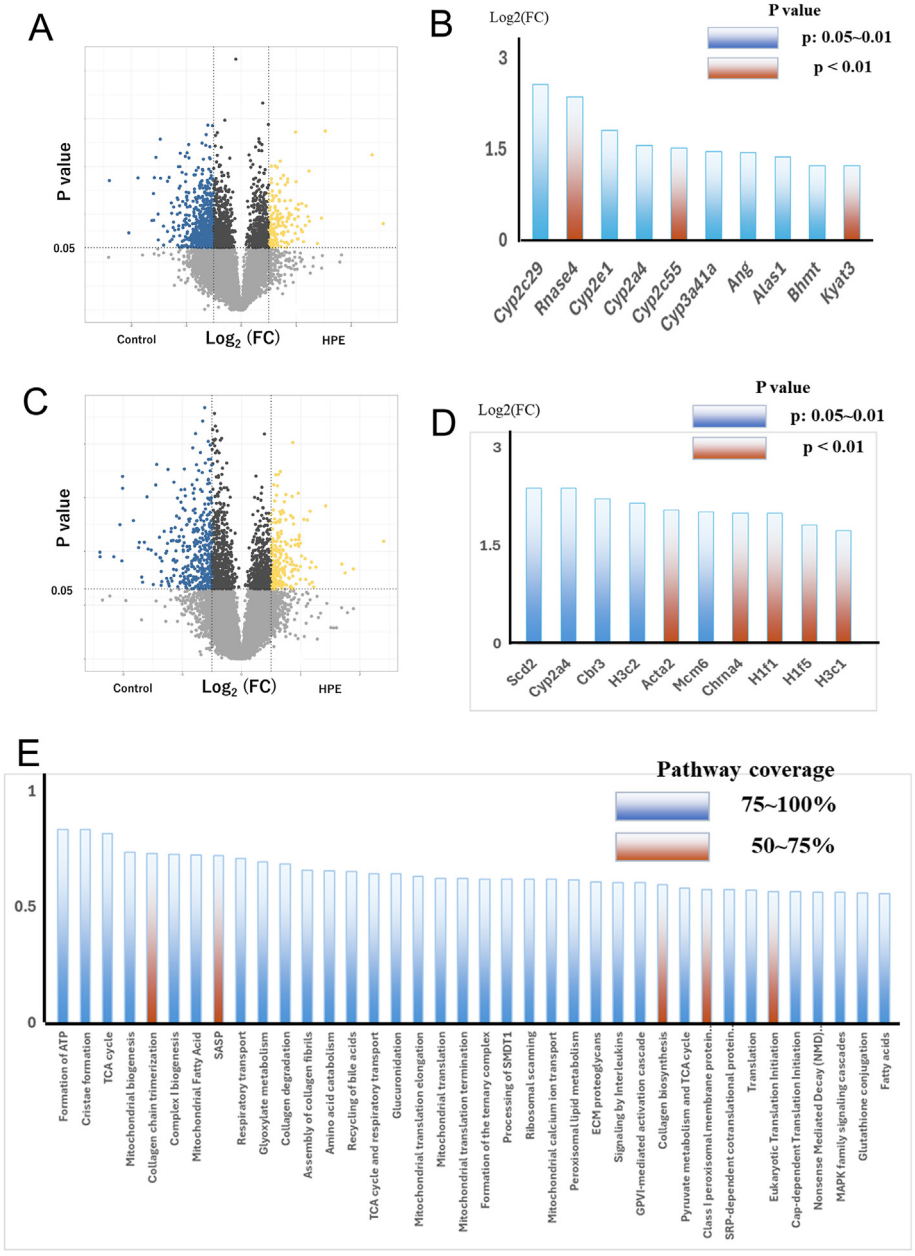

Fig. 4. Spatial transcriptomics with pathway analysis. (A) Volcano plot of the genes expressed in the ROI of the macrophage-rich regions in the control group compared to that in the human placental extract (HPE) administration group. Blue plots represent genes with $p < 0.05$ and high fold change (FC) toward the control group, whereas yellow plots represent genes with $p < 0.05$ and high FC toward the HPE administration group. (B) The top 10 enriched genes expressed in the macrophage-rich ROI. $p < 0.05$ is represented by the blue bar and $p < 0.01$ is represented by the brown bar. (C) Volcano plot of the genes expressed in the ROI of the macrophage-poor regions in the control group compared to that in the HPE group. Blue plots represent genes with $p < 0.05$ and high FC toward the control group, whereas yellow plots represent genes with $p < 0.05$ and high FC toward the HPE administration group. (D) The top 10 enriched genes expressed in the macrophage-poor ROI. $p < 0.05$ is represented by the blue bar and $p < 0.01$ is represented by the brown bar. (E) Pathway analysis of the genes expressed in the macrophage-rich ROI. Pathway intensity was scored between 0 and 1, and the top 40 factors were extracted in order of the absolute value of the score. Pathway coverage of 75–100 % is represented by a blue bar, while a coverage of 50–75 % is represented by a brown bar.

pathways related to mitochondrial function were enriched, which might reflect the anti-senescent effects.

Although we examined and discussed the effect of HPE on cirrhosis, this study had a few limitations. First, there may have been lot-to-lot differences in the therapeutic effects of HPE. Since the human placenta has individual differences, its quality may not be uniform. Company of HPE attempting to achieve uniformity in the manufacturing process; however, it may be necessary to identify which factors in HPE are truly effective against cirrhosis. Second, the amount of HPE administered to humans would be too large for clinical use. In this study, the dose of HPE administered to mice (approximately 30 g body weight) was 0.10 mL per subcutaneous injection. If this amount was administered to patients with cirrhosis (approximately 60 kg body weight), a subcutaneous injection of 200 mL per dose would be required, a limitation of the current form of HPE available. To solve these problems, it is important to identify effective factors in HPE and to administer only those factors (e.g., exosomes). Third, there is a lack of evidence of DNA synthesis via MAPK signaling, improvement of liver fibrosis, and hepatocyte regeneration. Regarding MAPK signaling, previous

reports suggested that HPE may promote cell proliferation through the upregulation of DNA synthesis [10]. In the pathway analysis of this study, MAPK signaling was involved but not to the main therapeutic effect of this study. Regarding liver fibrosis, although HPE tended to decrease the amount of liver fibrosis, no significant improvement was observed. Regarding hepatocyte regeneration, no significant upregulation was observed. For these points, the timeline of the analysis and the dose of HPE may not be the most appropriate, and it is important to accumulate further experiments.

In summary, this study showed that HPE may improve serum ALT levels through the effects of anti-inflammatory macrophages and the reduction of SASP in senescent cells (the mechanisms elucidated in this study are summarized in Graphical Abstract). Although some issues remain unresolved, HPE has many possibilities for therapeutic use. With further research, we believe that HPE will be beneficial for patients with cirrhosis.

Ethics statement

All animal experiments were approved by the Institutional Animal Care and Committee of Niigata University (SA01211).

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Declaration of competing interest

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2025.01.017.

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